Molecular Characterisation and Phylogenetics of Malaysian Green Arowana (*Scleropages formosus*) in Peninsular Malaysia

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The Asian arowana, *Scleropages formosus*, is one of the most highly prized ornamental fish in the Asian aquarium trade. The substantial decline of the populations due to over-exploitation and the destruction of their natural habitats have placed the species in the Red list of the International Union for Conservation of Nature and Natural Resources (IUCN). In Malaysia, the green variety of *S.formosus* is found only in Peninsula Malaysia, and none has been reported from East Malaysia. At present, only Lake Tasek Bera and Endau River are the major habitats in Peninsular Malaysia where wild populations of green arowana are thriving. The gold variety, however, is endemic to Bukit Merah Reservoir in Peninsula Malaysia and not found in East Malaysia. The present study analysed the molecular sequence variations and the phylogenetic relationship of natural populations of green arowana from both Lake Tasek Bera (10 specimens) and Endau River (5 specimens), together with 1 gold arowana from Bukit Merah, based on the mitochondrial DNA sequences of cytochrome b (627bp) and NADH dehydrogenase subunit 2 (ND2)-1041bp gene sequences. Variability for both genes were low (cytochome b- 4.5%, ND2- 2.8%) and mostly found at the third codon positions. Average base composition (A:C:G:T) for cytochrome- b was approximately 29%, 36%, 11% and 24% , whereas for ND2 it was 32%, 36%, 11% and 21%, respectively. Amino acid composition of the partial cytochrome-b sequences showed high percentage of leucine (19.56%) and phenylalanine (10.1%) residues, whereas ND2 sequences had high residues of leucine (19.88%) and threonine (15.55%). For both genes, the accumulation of genetic variation among the green arowana populations were mostly transitions, with few or almost no transversions. However, transversion rates began to increase when compared with other species and outgroups. A Neighbour Joining tree analysis of the combined genes using Kimura-2 parameter genetic distances revealed that genus Scleropages is monophyletic. *S.formosus* is also monophyletic, whereby the green arowana formed a monophyletic group. However, the populations of green arowana in Lake Tasik Bera and Endau Rompin could not be genetically differentiated. The gold arowana from Bukit Merah was more related to the red arowana from Indonesia than the Malaysian green arowana.

First ASIAHORCs Joint Symposium

MOLECULAR CHARACTERISATION AND PHYLOGENETICS OF MALAYSIAN GREEN AROWANA (*Scleropages formosus*) IN PENINSULAR MALAYSIA

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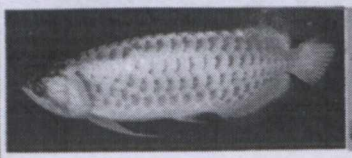
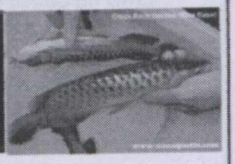
UNIVERSITI MALAYA

ASIAN AROWANA (*Scleropages formosus*)

- Also known as dragon fish or "kelisa" (Malay)
- Highly prized ornamental fish (China, Japan, South East Asia)
- Value as much as USD$ 200 per fingerling and between USD$ 1,000 and 2,000 for 30cm length (Pouyaud et al., 2003)
- In Malaysia, its popularity began as freshwater game fish (Stuart, 1953; Locke, 1956)
- Popular as aquarium fish in 1960s (Yue et al., 2004)


ASIAN AROWANA (*Scleropages formosus*)

- The body of the Asian arowana is compressed, dorsal and anal fin are placed on the rear half of the body.
- They have large scales with varieties of colours and two barbels protruding from the lower jaw.

SYSTEMATICS OF ASIAN AROWANA

- Ancient "bony-tongue" family (Osteoglossidae)
- Extant representatives restricted to freshwater (Nelson, 1994)
- Order Osteoglossiformes
  - Family Osteoglossidae (4 genera)
    - *Osteoglossum* (South America)
    - *Arapaima* (South America)
    - *Heterotis* (Africa)
    - *Scleropages* (Southeast Asia , Australia)




GENUS *Scleropages*

- Genus *Scleropages* (3 species):
  - Northern barramundi (*Scleropages jardinii)*
  - Spotted barramundi (*Scleropages leichardti*)
  - Asian arowana (*Scleropages formosus)*.
- Distribution pattern:
  - *Scleropages jardinii* (Northern Australia, Southern New Guinea)
  - *Scleropages leichardti* (Eastern Australia)
  - *Scleropages formosus* (Southeast Asia).

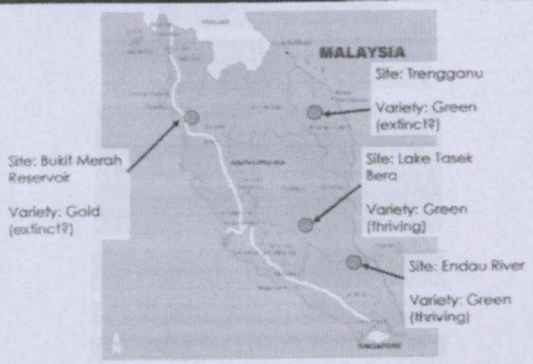
COLOUR VARIETIES OF *Scleropages formosus*

- Several types of *S. formosus* with different colour patterns inhabit separate regions of Southeast Asia
- Distributed into freshwater habitats during the Pleistocene glacial ages. (Goh & Chua, 1999)
- *S.formosus* have four natural colour varieties (Goh & Chua, 1999, Pouyaud et al., 2003):-
  - ❑Cross Back Golden -**Peninsular Malaysia**
  - ❑Super Red - **West Kalimantan (Indonesia)**
  - ❑Red Tail Golden - **Pekan Baru (Indonesia)**
  - ❑Green – **Peninsular Malaysia, Indonesia, Myanmar, Thailand**

DISTRIBUTION OF *S.formosus* IN PENISULAR MALAYSIA

- Green and gold varieties only in the Peninsular Malaysia
- None has been reported from East Malaysia, Sabah and Sarawak (Suleiman, 2003)
- Green variety has been reported in some area in Peninsular Malaysia:-
  - ❑Lake Tasek Bera (Pahang)
  - ❑Endau River (Johor)
  - ❑Terengganu drainage
- Gold variety is believed endemic to Bukit Merah Reservoir, in Perak, Malaysia. (Suleiman , 2003)
- Declining populations due to over-exploitations and destruction of their natural habitats.
- Placement of the species in the Red List of IUCN and Appendix I in CITES.

DISTRIBUTION OF *S.formosus* IN PENINSULAR MALAYSIA



MOLECULAR GENETIC STUDIES

- Molecular genetic techniques have been applied in captive breeding and conservation programs of this endangered species.
- Some of the techniques were based on RFLP, RAPD, AFLP, DNA fingerprinting, and microsatellite analysis.
- However, studies were more focused on captive stocks.
- Very few studies investigate the genetic variation of the natural and wild populations.

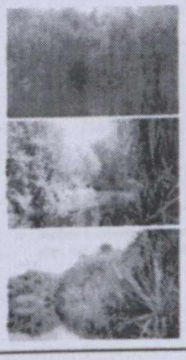
OBJECTIVES

- To describe the mitochondrial DNA sequence of natural population of green arowana from Lake Tasek Bera and Endau River (5'-end of partial *cyt-b* and complete *ND2* genes).
- To determine the accumulation of substitutions.
- To reconstruct the phylogenetic tree (*cyt-b* and *ND2* genes).
- To determine whether Tasek Bera population is different from Endau River population.

MATERIALS AND METHODS

- Collection of samples (16 specimens) from:
  - ❑Lake Tasek Bera (Pahang)
  - ❑Endau river (Johor)
- Total of 23 sequences :
  - ❑15 green (10 Tasik Bera and 5 Endau Rompin)
  - ❑1 gold (Bukit Merah)
  - ❑2 red (Indonesian) by Kumazawa et al, (2004).
  - ❑5 outgroup sequences (*Arapaima gigas, Osteoglossum bicirrhosum, Osteoglossum ferreirai, Scleropages jardinii* and *Scleropages leichardti*).

LAKE TASEK BERA

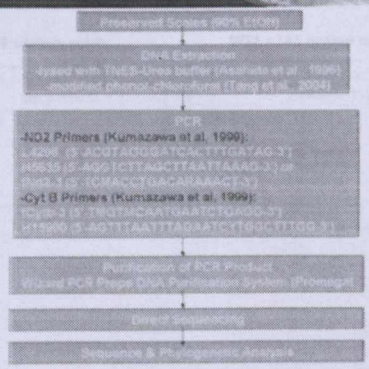


- Peat swamp (Black water)
- Water is dark, tea coloured, and is black as seen by reflected light
- Acidic (pH4.3-6.45)
- Less water flow
- Vegetation :
  - ❑*Pandanus heliocoptus*
  - ❑*Lepironia articulata*

ENDAU RIVER

- Riverine system with pools
- Variable flow of water current
- Acidic (pH5.47-6.97)
- Typical rainforest vegetation

PCR AND SEQUENCING



MITOCHONDRIAL DNA SEQUENCES

Species name	Location (Species ID)	No of specimen	Accession No. (CytB)	Accession No. (ND2)
S. formosus	Endau Rompin	5	DQ864671-5	DQ864684-5 DQ147907-8 DQ147902
S. formosus	Bukit Merah	1	DQ864676	DQ147909
S. formosus	Tasik Bera	10	DQ864661-70	DQ864677-83 DQ864686 DQ147901 DQ147906
S. formosus	Indonesia	2	AB035234-5	AB035221
S. jardinii	Northern Australia	1	AB035236	AB035223
S. leichardti	Eastern Australia	1	AB035237	AB035224
O. bicirrhosum	South America	1	AB035238	AB035225
O. ferreirai	South America	1	AB035239	AB032526
Arapaima gigas	South America	1	AB035241	AB032528

BASE COMPOSITION

Base composition of cyt-b sequences

Species	T(U)	C	A	G
S. formosus	24.3*	35.8*	28.9*	11.0*
S. jardinii	26.5	34.9	25.7	12.9
S. leichardti	26.6	34.9	26.3	12.1
O. bicirrhosum	29.3	29.8	29.7	11.2
O. ferreirai	28.7	30.5	30.3	10.5
A. gigas	30.5	30.6	28.1	10.8

*average of 15 *S. formosus* (green arowana) sequences

BASE COMPOSITION

Base composition of ND2 sequences

Species	T(U)	C	A	G
S. formosus	20.9*	35.8*	31.7*	11.6*
S. jardinii	21.8	34.1	29.8	14.3
S. leichardti	21.8	34.6	32.5	11.1
O. bicirrhosum	23.2	32.2	34.5	10.1
O. ferreirai	23.3	32.0	34.8	9.9
A. gigas	27.2	29.9	32.2	9.7

*average of 15 *S. formosus* (green arowana) sequences

SEQUENCE VARIATION

cyt-b

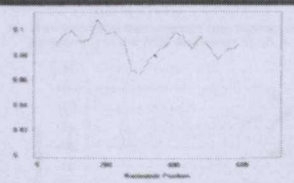
Type of site	Base frequency	(%)
Conserved	599	95.5
Variable	28	4.5
Singleton	13	2.1
Parsimony info	15	2.4

ND2

Type of site	Base frequency	(%)
Conserved	1012	97.2
Variable	29	2.8
Singleton	9	0.9
Parsimony info	20	1.9

Nucleotide frequency of variable sites of Cyt B (627bp) and ND2 (1041bp) from the alignment of 18 *S. formosus sequences*

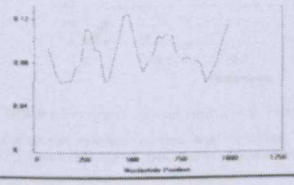
DISTRIBUTION OF SEQUENCE VARIATION



cyt b

Most variation found at position 50-250 and 400-600.

Average Pi nucleotide diversity= 0.088



ND2

Most variation found at position 80-90, 250-300, 470-510, 625-700 and 1000.

Average Pi nucleotide diversity= 0.09

AMINO ACID SEQUENCE VARIATION

Cyt-b=209 amino acids

Type of site	Amino acid frequency	(%)
Conserved	202	96.7
Variable	7	3.4
Singleton	6	2.9
Parsimony info	1	0.5

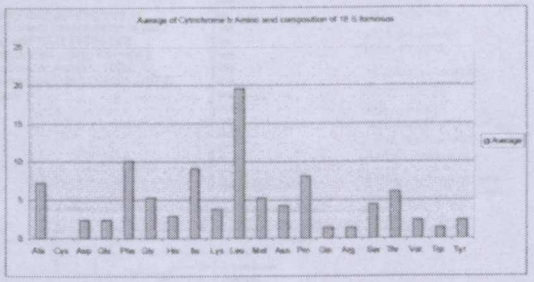
ND2 =347 amino acids

Type of site	Amino acid frequency	(%)
Conserved	339	97.7
Variable	8	2.3
Singleton	5	1.44
Parsimony info	3	0.9

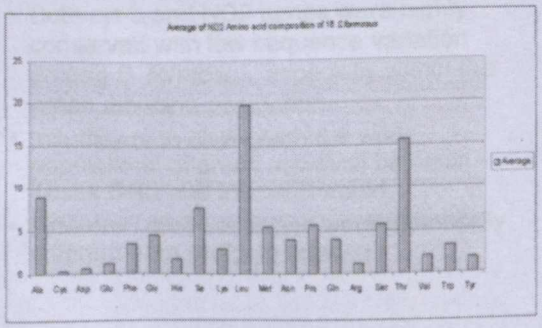
AMINO ACID COMPOSITION

- Amino acid composition of partial cyt-b sequences showed high percentage of leucine (19.56%) and phenylalanine (10.1%) residues.
- ND2 sequences also had high percentage of leucine (19.88%) and threonine (15.55%) residues.

AMINO ACID COMPOSITION

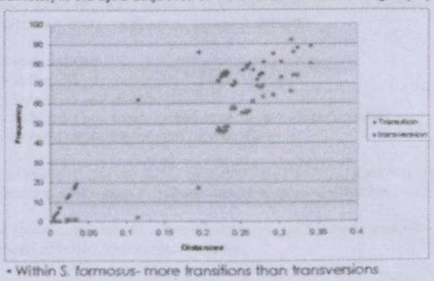


AMINO ACID SEQUENCE VARIATION



SUBSTITUTION PATTERN

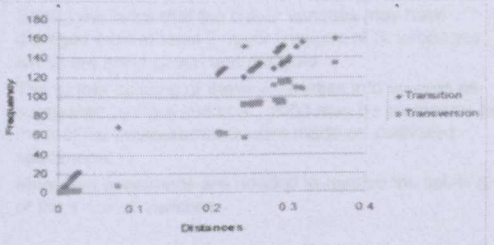
- Transition and transversion rate against the genetic distance (Kimura-2-Parameter) in the cyt-b sequence of 18 *S. formosus* and 5 outgroup species.



- Within *S. formosus*- more transitions than transversions
- Between genus/species- high level of transitions and transversions. Members of Osteoglossidae are highly divergent.

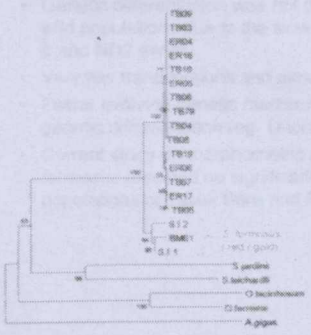
SUBSTITUTION PATTERN

- Transition and transversion rate against the genetic distance (Kimura-2-parameter) in the ND2 sequence of 18 *S. formosus* and 5 outgroup species



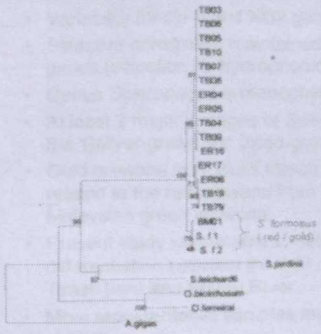
- Similar substitution pattern as in cyt-b, but with higher substitution frequencies.
- For closely related species, the transition/ transversion ratio approximately 10:1. More divergent species have transitions/transversion ratio of 2:1.

PHYLOGENETIC TREE



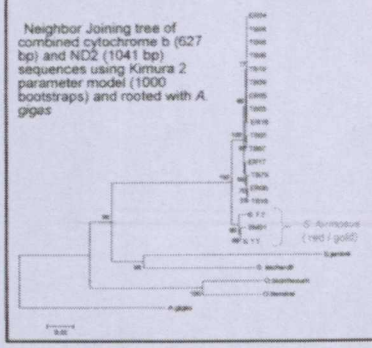
Neighbor Joining tree of 23 cytochrome b sequences (627 bp) using Kimura 2 parameter model (1000 bootstraps) and rooted with *A. gigas*

PHYLOGENETIC TREE



Neighbor Joining tree of 23 ND2 sequences (1041 bp) using Kimura 2 parameter model (1000 bootstraps) and rooted with *A. gigas*

PHYLOGENETIC TREE



Neighbor Joining tree of combined cytochrome b (627 bp) and ND2 (1041 bp) sequences using Kimura 2 parameter model (1000 bootstraps) and rooted with *A. gigas*

- Genus Scleropages is monophyletic
- *S. formosus* is monophyletic
- Green arowana is monophyletic
- No genetic differentiation between Tasek Bera and Endau Rompin populations
- Gold arowana more related to red arowana

DISCUSSION

- Both cyt-b and ND2 genes were highly conserved with low sequence variation among *S. formosus*, especially within the green arowana population.
- Insufficient to distinguish the wild populations of green arowana between Tasek Bera and Endau Rompin.
- However, green arowana were genetically different from red/gold arowana.

GENETICALLY DIFFERENT COLOUR VARIETIES?

- Pouyaud *et al.*, 2003 have suggested that colour varieties of *S. formosus* may represent different species.
- The species are
  - *Scleropages formosus* (Green varieties)
  - *Scleropages aureus* (Silver)
  - *Scleropages macrocephalus* (Red-tailed Golden)
  - *Scleropages legendrei* (Super Red)
- His analysis of partial cyt-b sequences (3'end)suggests a close relationship between the silver and the green varieties.
- Our study suggests that the Malaysian gold variety appears to be more related to the red varieties.

GENETICALLY DIFFERENT COLOUR VARIETIES?

- Strong evidence that the colour varieties may have diverged from at least 2 major lineages of Scleropages which are silver-green and red-gold.
- The further splitting of these 4 varieties into species as suggested by Pouyaud et al., 2003 may be premature as most of his measurements were made on cultivated specimens.
- More wild specimens are needed to resolve the splitting of the 4 colour varieties.

WILD POPULATIONS OF LAKE TASEK BERA AND ENDAU RIVER

- Genetic differentiation was not detected between the wild populations due to the slow mutation rate of the cyt-b and ND2 genes.
- Very few transversions and almost saturated transitions.
- Faster evolving genetic marker may potentially reveal genetic differentiation (eg- D-loop).
- Current study on morphometric and meristic of *S. formosus* revealed no significant difference between wild populations of Tasek Bera and Endau River.

CONCLUSION

- Variability for cyt-b and ND2 genes were low.
- Selective constraints maintained the function of the genes (selection for hydrophobic amino acid residues).
- Genus *Scleropages* is monophyletic.
- At least 2 major lineages of *Scleropages* were identified, the 1)silver-green and 2)red-gold lineages.
- Gold arowana from Bukit Merah Reservoir was more related to the red arowana from Indonesia than the Malaysian green arowana.
- Present study was unable to detect genetic differentiation between the wild populations of Lake Tasek Bera and Endau River.
- More representative samples are required for future studies.